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Comparison of Subgingival Bacterial Sampling With Oral Lavage for Detection and Quantification of Periodontal Pathogens by Real-Time Polymerase Chain Reaction

Khalil Boutaga,* Paul H.M. Savelkoul,† Edwin G. Winkel,‡§ and Arie J. van Winkelhoff*

Background: Saliva has been studied for the presence of subgingival pathogens in periodontitis patients. With the anaerobic culture technique, the discrepancy between salivary recovery and subgingival presence has been significant, which makes this approach not suitable for practical use in the microbial diagnosis of periodontitis patients. The real-time polymerase chain reaction (PCR) technique represents a very sensitive technique to detect and quantify bacterial pathogens. The aim of the study was to compare the presence and numbers of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Prevotella intermedia*, and *Micromonas micros* in subgingival plaque and mouthwash samples by the anaerobic culture and real-time PCR techniques.

Methods: Pooled subgingival plaque samples and 10-ml mouthwash samples were collected from 21 adult patients with periodontitis and analyzed by quantitative anaerobic culture and real-time PCR for *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythensis*, *P. intermedia*, and *M. micros*.

Results: The detection frequency of *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythensis* in subgingival plaque was identical by culture and real-time PCR and was higher for *P. intermedia* and *M. micros* by real-time PCR. The highest detection frequencies for the target bacteria were found in mouthwash samples by real-time PCR. The additional value of the real-time PCR to detect target bacteria was 38% for *P. gingivalis*, 73% for *T. forsythensis*, 77% for *P. intermedia*, and 71% for *M. micros*. The sensitivity to detect target species in mouthwash by real-time PCR was 100% for all test species except for *P. intermedia* (93.8%).

Conclusions: Rapid detection and quantification of periodontal pathogens in mouthwash samples are possible by real-time PCR. The procedure is significantly less time-consuming than subgingival sampling with paper points. This approach to detect major periodontal pathogens in mouthwash samples may simplify microbial diagnosis in periodontitis patients and may be used to monitor periodontal treatment. *J Periodontol* 2007;78:79-86.

KEY WORDS

Dental plaque; mouthwash; periodontitis; polymerase chain reaction.

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Bacterial plaque is considered the principle etiological factor in the onset and progression of periodontitis.^{1,2} *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Prevotella intermedia*, and *Micromonas micros* are strong markers of periodontitis in adults,^{2,3} and these species have been linked to the progression of the disease.⁴ In the microbiological diagnosis of periodontal diseases, subgingival plaque is commonly used to detect and quantify bacterial species. Curets, dental floss, and paper points have been used to sample subgingival plaque. The technique used may influence the outcome of the microbiological analysis.^{5,6} Also, the number of sample sites is essential, especially when samples are pooled.⁷ Microbial subgingival sampling is time consuming and involves the selection of sampling sites, isolation and drying of these sites, and sampling of the subgingival area.

The use of saliva for diagnostic purposes has been the subject of considerable research.⁸ Saliva is easy to obtain and contains bacteria from different oral sites including oral mucosal sites and supra- and subgingival plaque. Consequently, studies have evaluated the presence and levels of bacteria in saliva in relation to the periodontal status. Asikainen et al.⁹ used the culture technique to investigate the presence of *A. actinomycetemcomitans* in stimulated and unstimulated saliva samples and found these pathogens in 69.9% and 35.9% of the samples, respectively, when *A. actinomycetemcomitans* was detected subgingivally. Umeda et al.¹⁰ used the polymerase chain reaction (PCR) technique to detect periodontal pathogens in pooled subgingival plaque and whole saliva samples. They found that whole saliva samples were more frequently positive for *P. gingivalis* and *Treponema denticola* than subgingival plaque samples, whereas saliva samples underestimated the presence of *A. actinomycetemcomitans* and *T. forsythensis* compared to subgingival samples.

Real-time PCR is a relatively new molecular technique to detect and quantify periodontal pathogens and has been used for oral samples by several authors.¹¹⁻¹⁷ The sensitivity of this technique allows the detection of very small numbers of pathogens. Salivary microbiological diagnosis provides a non-invasive, inexpensive, and rapid technique for the detection and quantification of periodontal pathogens.

We hypothesized that oral mouthwash samples (oral lavage) can be used to detect and quantify subgingival pathogens, providing a sensitive detection technique. Therefore, the purpose of this study was to compare the frequency of detection and the number of pathogens in subgingival plaque samples and mouthwash samples by anaerobic culture and real-time PCR techniques.

MATERIALS AND METHODS

Study Population

A study group of 21 adult patients with untreated periodontitis was selected after visiting the Clinic for Periodontology and Implantology Amsterdam. Patients had periodontal pockets >5 mm with clinical attachment loss that showed bleeding upon pocket probing in all four quadrants of the dentition as determined at the baseline visit. Patients had not used antibiotics in the past 3 months. Subjects were verbally informed about the purpose of the investigation. Patients were enrolled in the study from January to March 2005. Patients participated in the study on the basis of informed consent. The study was approved by the Medical Ethical Committee of the Vrije University Amsterdam. Table 1 shows the demographic and periodontal characteristics of the study subjects.

Sampling

At the second visit to the dental office (at least 2 weeks after the baseline measurements), patients were asked to rinse with 10 ml sterile saline for 30 seconds. The deepest pocket in each quadrant of the dentition was used as the subgingival sample site. The level of supragingival plaque at these sites was evaluated at the gingival margin by means of a periodontal probe (2 = visible plaque; 1 = plaque on periodontal probe; and 0 = no visible plaque on periodontal probe). Sample sites were isolated with cotton rolls, and supragingival plaque was carefully removed with curets, and the sites were air dried. A subgingival plaque sample was taken by two sterile paper points that were consecutively inserted into the periodontal pocket and removed after 10 seconds. All paper points were transferred to a vial containing 1.5 ml reduced transport medium.¹⁸ Mouthwash samples and pooled subgingival plaque samples were processed for anaerobic microbiological analysis and real-time PCR within 4 hours.

Microbiological Procedures

Samples were vortexed for 2 minutes and split: 100 μ l was used to prepare 10-fold serial dilutions in sterile phosphate buffered saline (PBS) and used for culture, and 100 μ l was stored at -20°C and used for real-time PCR testing within 3 weeks. For anaerobic culture, 100 μ l of appropriate dilutions were plated on blood agar plates that were supplemented with horse blood (5% volume/volume [v/v]), hemin (5 mg/l), and menadione (1 mg/l) and incubated in 80% N₂, 10% H₂, and 10% CO₂ at 37°C for up to 14 days. *A. actinomycetemcomitans* was grown on trypticase-soy with serum, bacitracin, and vancomycin trypticase (TSBV) plates and incubated at 37°C in air + 5% CO₂ for 3 days. Identification was done as described by Boutaga et al.^{13||}

|| No. 2, Oxoid, Basingstoke, U.K.

Table 1.**Demographic Data and Mean (SD) Clinical Parameters of Sampling Sites**

Characteristics	N	Mean (SD)	Range
Males	4	—	—
Females	17	—	—
Age (years)	—	44.80 (9.10)	32-63
PI	—	0.91 (0.62)	0-2
BOP	—	1.68 (0.40)	0-2
PD (mm)	—	6.48 (1.04)	4.75-8.5
Attachment loss (mm)	—	4.45 (3.68)	0-8.5

— = not applicable; PI = plaque index; BOP = bleeding on probing; PD = probing depth.

The strains used in this study were as follows: *P. gingivalis* (W83), *T. forsythensis* (clinical isolate), *A. actinomycetemcomitans* (National Collection of Type Cultures [NCTC] 9710), *P. intermedia* (American Type Culture Collection [ATCC] 25611), and *M. micros* (clinical isolate). Reference strains were grown as recommended by the ATCC. Determination of the number of the total colony forming units (CFU) per milliliter of the bacterial suspensions was performed by growing the bacteria 2 to 3 days in brain-heart infusion (BHI) supplemented with 5 mg/l hemin and 1 mg/l menadione, and plating serial dilutions as described by Boutaga et al.¹³

Quantitative Real-Time PCR

DNA isolation from plaque samples, mouthwash, and bacterial reference cultures. From plaque samples, mouthwash, and bacterial culture dilutions, 100 μ l was used for automated DNA extraction and purification with a DNA isolation kit.[¶] The protocol included a 1-hour pretreatment with proteinase K (20 mg/ml) at 56°C and 10 minutes deactivation at 100°C. Afterwards, isolation DNA was eluted in 100 μ l elution buffer.

Real-time PCR. The primer/probe sets and real-time PCR conditions were performed as described previously.¹³ Briefly, real-time PCR amplification was performed in a total reaction mixture volume of 25 μ l. The reaction mixtures contained 12.5 μ l 2 \times universal PCR master mix (PCR buffer, deoxynucleoside triphosphates, DNA polymerase,[#] reference signal [6-carboxy-X-rhodamine], uracil *N*-glycosylase, and MgCl₂),** 300 to 900 nM pathogen-specific primer, 50 to 100 nM pathogen-specific probe, and 5 μ l purified DNA from plaque samples. The samples were subjected to an initial amplification cycle of 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles at

95°C for 15 seconds and 60°C for 1 minute. The data were analyzed with sequence detection software.^{††}

For quantification, the results from unknown plaque samples were projected on the counted pure-culture standard curves of the target bacteria.

The possible inhibition of the real-time PCR was determined by comparing the results of spiked amplifications to the original samples. Each sample was spiked with 1,000 CFU *Escherichia coli* DH5 α (i.e., 50 CFU equivalents/PCR) before DNA isolation. In addition, negative amplifications were spiked with the target bacteria in numbers close to the threshold line. The primers and probe used for amplification of *E. coli* have been described by Huijsdens et al.¹⁹

RESULTS

The prevalence of the target bacteria in subgingival plaque and mouthwash samples based on the anaerobic culture and RT-PCR techniques is shown in Figure 1.

Culture

Subgingival plaque samples were clearly more often culture positive for *P. gingivalis*, *T. forsythensis*, *P. intermedia*, and *M. micros* than culture mouthwash samples. The differences in prevalence ranged between 23.8% for *P. gingivalis* to 57.1% for *T. forsythensis*. *A. actinomycetemcomitans* was the least frequently (<10%) recovered species with both techniques from subgingival plaque. For *A. actinomycetemcomitans*, mouthwash samples were more often culture positive.

Real-Time PCR

Mouthwash samples were more often real-time PCR positive than subgingival plaque samples for all target species except for *M. micros* for which the prevalence was 100% in both mouthwash and plaque samples. It was striking that the prevalence of *A. actinomycetemcomitans* (9%), *P. gingivalis* (33.3%), and *T. forsythensis* (85.7%) in real-time PCR plaque-tested samples was identical to culture plaque-tested samples. The frequency of detection of *P. gingivalis* in mouthwash samples increased from 9.5% by culture to 47.6% by real-time PCR. The frequency of detection of *T. forsythensis* in mouthwash increased from 28.6% by culture to 100% by real-time PCR. The prevalence of *P. intermedia* in mouthwash showed a marked difference: 4% by culture to 82% by real-time PCR. *M. micros* was also more frequently detected in mouthwash by real-time PCR than by culture (76.2% versus 28.6%).

¶ MagNA Pure DNA Isolation Kit III, Roche Molecular Diagnostics, Almere, The Netherlands.

AmpliTaq Gold, Applied Biosystems, Foster City, CA.

** TaqMan, Applied Biosystems.

†† ABI 7000, Applied Biosystems.

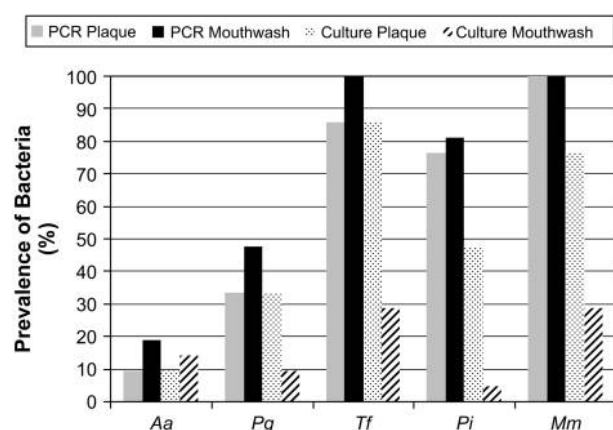


Figure 1.

Prevalence (%) of *A. actinomycetemcomitans* (Aa), *P. gingivalis* (Pg), *T. forsythensis* (Tf), *P. intermedia* (Pi), and *M. micros* (Mm) in subgingival plaque samples and mouthwash by anaerobic culture and real-time PCR.

Numbers of Bacteria

The results of the quantification of tested bacterial species in mouthwash and subgingival plaque samples for each method are summarized in Table 2. Subgingival plaque samples yielded more bacterial cells of each species than mouthwash samples by culture and real-time PCR. The mean CFU counts of the tested species by real-time PCR and culture did not differ more than one log for plaque samples and between two and four logs for mouthwash samples.

Figures 2 and 3 depict the prevalence per subject of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythensis*, *P. intermedia*, and *M. micros* by real-time PCR and culture, respectively. The results obtained by real-time PCR in mouthwash samples matched the results obtained in subgingival plaque samples for

90%, 86%, 86%, 86%, and 95% of the subjects infected with *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythensis*, *P. intermedia*, and *M. micros*, respectively. The results obtained by anaerobic culture in mouthwash samples matched the results obtained in subgingival plaque samples for 95%, 76%, 33%, 57%, and 43% of the subjects infected with *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythensis*, *P. intermedia*, and *M. micros*, respectively.

In Table 3, the calculated sensitivity and specificity values are presented with the subgingival plaque samples as reference criteria. With real-time PCR, the sensitivity was 100% to detect *P. gingivalis*, *T. forsythensis*, and *M. micros* in the mouthwash and 93.8% to detect *P. intermedia*. For culture, the sensitivity to detect target bacteria in mouthwash when detected subgingivally was <35% except for *A. actinomycetemcomitans*, which was 100%. The specificity was also calculated and varied between 0% and 89.5% by real-time PCR and between 66.7% and 100% by anaerobic culture.

Finally, a linear correlation calculated on the basis of the quantitative results for mouthwash and subgingival plaque samples by real-time PCR ($R^2 = 0.202$ to 0.601) and anaerobic culture ($R^2 = 0.262$ to 0.554) showed that there is a reasonably good correlation between sample types at the quantitative level.

DISCUSSION

The presence and numbers of periodontal pathogens in plaque and saliva samples have been studied using culture, immunoassays, DNA probes, and PCR techniques.^{16,20-24} These methods have limited specificity and sensitivity. Real-time PCR, with species-specific primers and probe, represents a specific, sensitive, and quantitative tool to study periodontal pathogens more accurately and is able to detect as little as a

Table 2.

Number of Bacterial Cells per Milliliter for Each Species Tested by Anaerobic Culture and Real-Time PCR

Anaerobic Culture and Real-Time PCR Detected	Mean CFU Count			
	Culture	Real-Time PCR	Culture	Real-Time PCR
	Subgingival		Mouthwash	
<i>A. actinomycetemcomitans</i>	3.60E+03	2.98E+04	1.21E+03	1.81E+03
<i>P. gingivalis</i>	1.37E+06	4.25E+05	7.14E+04	6.50E+04
<i>T. forsythensis</i>	1.70E+06	2.60E+07	7.95E+04	1.43E+06
<i>P. intermedia</i>	2.14E+06	3.30E+06	1.43E+02	2.59E+06
<i>M. micros</i>	2.99E+06	2.29E+07	6.57E+04	8.67E+06

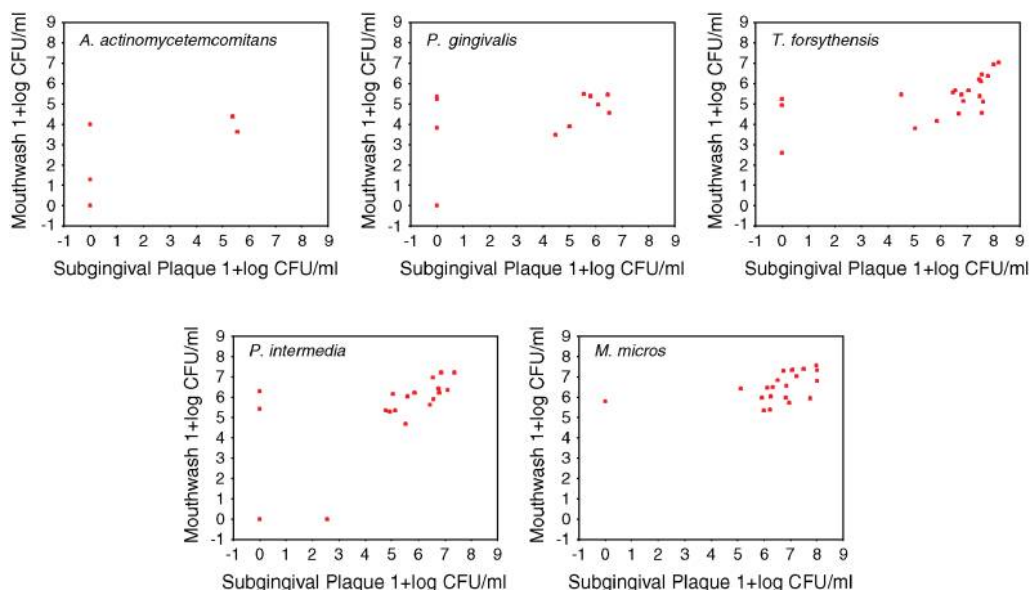


Figure 2.

Scatter plot showing the differences and correlations between subgingival plaque and mouthwash samples by real-time PCR. Data of negative mouthwash and positive plaque samples fall near the x axis. Data suggesting the additional value of the mouthwash samples fall near the y axis.

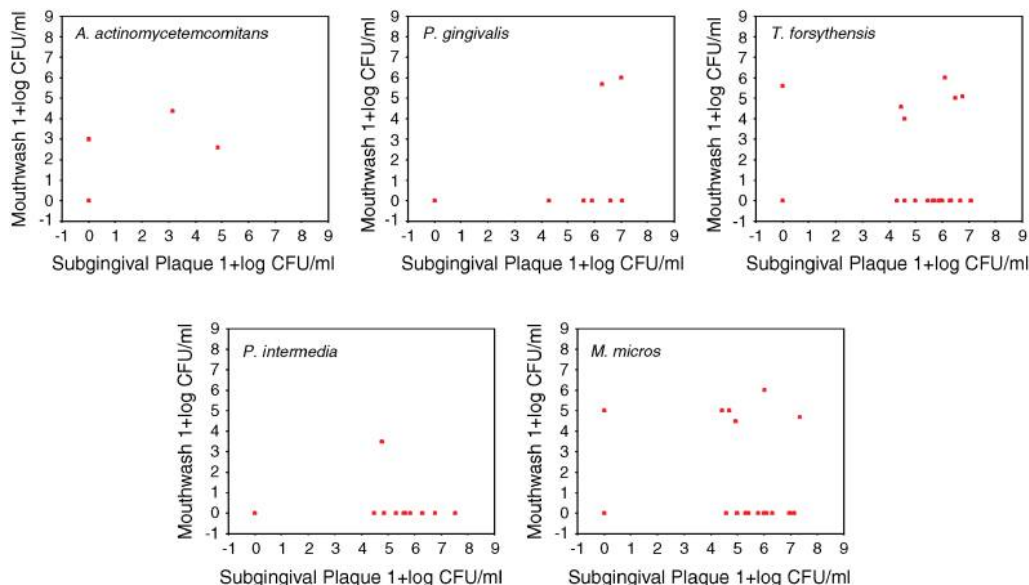


Figure 3.

Scatter plot showing the differences and correlations between subgingival plaque and mouthwash samples by anaerobic culture. Data of negative mouthwash and positive plaque samples fall near the x axis. Data suggesting the additional value of the mouthwash samples fall near the y axis.

tection frequency and numbers by real-time PCR. The reason for this study was to assess whether a mouthwash can be used to detect subgingival pathogens based on the improved sensitivity of the real-time PCR.

In this study, subgingival plaque cultures were superior to mouthwash cultures to detect the target bacteria.

We found mouthwash samples more often positive by real-time PCR for the target bacteria than subgingival plaque samples, except for *M. micros* for which the prevalence was equal. These results contrast with those reported by Testa et al.,²⁶ who examined the correlation between the number and type of bacteria from periodontal pockets >4 mm deep and saliva using bacterial culture and microscopy. They found no significant differences between counts of the microorganisms in saliva and in the subgingival plaque samples.

Umeda et al.¹⁰ used PCR and found saliva superior compared to subgingival plaque for the detection of *P. gingivalis*, *T. forsythensis*, and *P. intermedia*. In their study, the prevalence of *P. gingivalis*, *T. forsythensis*, and *P. intermedia* in subgingival plaque was 48%,

single bacterial cell.^{11-13,22,25} The aim of the present study was to determine the detection frequency and numbers of a set of periodontal bacterial species in subgingival plaque and in mouthwash using culture techniques and to compare these results with the de-

44%, and 31%, respectively, and 48%, 55%, and 43% in saliva samples, respectively. *A. actinomycetemcomitans* was detected less frequently in whole saliva (20%) compared to periodontal pocket samples (27%). For some authors,^{10,27,28} the concurrent

Table 3.**Comparison of Detection of Five Periodontal Pathogens in Mouthwash and Subgingival Plaque in 21 Periodontitis Patients**

Real-Time PCR Microorganisms	Mouthwash			Sensitivity (%)	Specificity (%)
	+	–	Total		
Subgingival plaque					
<i>A. actinomycetemcomitans</i>					
+	2	0	2	100	89.5
–	2	17	19		
<i>P. gingivalis</i>					
+	7	0	7	100	78.6
–	3	11	14		
<i>T. forsythensis</i>					
+	18	0	18	100	ND
–	3	0	3		
<i>P. intermedia</i>					
+	15	1	16	93.8	75
–	2	3	5		
<i>M. micros</i>					
+	20	0	20	100	ND
–	1	0	1		
Anaerobic Culture Microorganisms	Mouthwash			Sensitivity (%)	Specificity (%)
	+	–	Total		
Subgingival plaque					
<i>A. actinomycetemcomitans</i>					
+	2	0	2	100	94.7
–	1	18	19		
<i>P. gingivalis</i>					
+	2	5	7	29	100
–	0	14	14		
<i>T. forsythensis</i>					
+	5	13	18	28	66.7
–	1	2	3		
<i>P. intermedia</i>					
+	1	9	10	10	100
–	0	11	11		
<i>M. micros</i>					
+	5	11	16	31	80
–	1	4	5		

ND = not determined.

presence of periodontal bacteria in saliva and subgingival plaque and the presence of the same genetic clonal types in both samples suggest dissemination of bacteria from periodontal pockets into saliva. These observations support the use of saliva or a mouthwash for microbiological sampling in periodontics.^{9,10,29,30} Several investigators have suggested that oral colonization by *A. actinomycetemcomitans* could be successfully determined by using oral mucosa or saliva samples.^{31,32} Cortelli et al.³³ studied the prevalence

of *A. actinomycetemcomitans* in subgingival plaque, unstimulated saliva, and mucosal sites (dorsum of the tongue and buccal mucosa) in patients with severe periodontitis using a selective-culture technique. They detected *A. actinomycetemcomitans* in 63% of the pocket samples, 56% of the saliva samples, and 45% of the mucosal samples. The authors suggested that saliva samples could be an effective alternative method for pooled subgingival plaque samples to detect *A. actinomycetemcomitans*. In our opinion,

the discrepancy between subgingival, mucosal, and salivary detection is too large for the reliable detection of this pathogen in extracrevicular samples by culture. Mager et al.³⁴ also found that saliva can be used to detect *A. actinomycetemcomitans* but may not be suitable for detection of *T. forsythensis*.

The number of *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythensis*, *P. intermedia*, and *M. micros* in subgingival samples determined by real-time PCR correlated well with the numbers of CFU determined by culture (Table 2). However, in mouthwash samples, the number of *T. forsythensis*, *P. intermedia*, and *M. micros* showed a difference between real-time PCR and culture. This can be explained by insufficient homogenization of the mouthwash samples, salivary factors affecting the growth of different isolates, and the presence of antagonistic bacterial species. Consequently, the higher bacteria counts by PCR may be due to the fact that the real-time PCR detects viable and dead cells in clinical specimens.

The sensitivity of the real-time PCR to detect pathogens in mouthwash samples was very good for the tested species and ranged from 93% to 100%. *P. intermedia* was missed in 4.8% of the mouthwash samples (one sample). This real-time PCR false-negative finding may also be explained by a misdiagnosis by culture. The sensitivity of the anaerobic culture was significantly lower, ranging from 10% to 31%. However, for *A. actinomycetemcomitans*, the sensitivity was 100%. This may be related to the fact that *A. actinomycetemcomitans* is not a strict anaerobic bacterium.

The specificity of the real-time PCR to detect *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* in mouthwash samples ranged from 75% to 89%. For *T. forsythensis* and *M. micros*, specificity could not be calculated due to the negative subgingival plaque and negative mouthwash results. When anaerobic culture was used, the specificity ranged between 66% and 100% for detection and quantification of the target pathogens.

Saliva also contains proteolytic enzymes coming from the host and from oral microorganisms, which may affect the stability of DNA amplifications. Despite these disadvantages, the popularity of oral lavage for bacterial diagnostic tests is increasing. However, this study showed that mouthwash samples, in combination with real-time PCR, are useful for both qualitative and quantitative tests and better than subgingival plaque.

CONCLUSIONS

In this study, we found that microbial analyses of mouthwash samples from patients with periodontitis by real-time PCR can replace subgingival sampling. Mouthwash sampling is a non-invasive technique that

is easy to obtain, inexpensive, and cost effective. This approach can be used for screening large populations and monitoring antimicrobial treatments. For patients, the non-invasive mouthwash technique reduces anxiety and discomfort.

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